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Antigen-Specific Suppression of a Primed Immune Response by Dendritic Cells Mediated by Regulatory T Cells Secreting Interleukin-10

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Summary

Antigen-specific suppression of a previously primed immune response is a major challenge for immunotherapy of autoimmune disease. RelB activation is required for myeloid DC differentiation. Here, we show that antigen-exposed DCs in which RelB function is inhibited lack cell surface CD40, prevent priming of immunity, and suppress previously primed immune responses. DCs generated from CD40-deficient mice similarly confer suppression. Regulatory $CD4^+$ T cells induced by the DCs transfer antigen-specific "infectious" tolerance to primed recipients in an interleukin-10-dependent fashion. Thus CD40, regulated by RelB activity, determines the consequences of antigen presentation by myeloid DCs. These observations have significance for autoimmune immunotherapy and suggest a mechanism by which peripheral tolerance might be constitutively maintained by $RelB^-$ $CD40^-$ DCs.

Introduction

Antigen-specific suppression of a previously primed immune response is a major challenge for immunotherapy of autoimmune disease. Blockade of products of the innate immune system, including TNF-α and IL-1β, produces dramatic anti-destructive clinical effects in the autoimmune disease rheumatoid arthritis (RA) (reviewed by Feldmann, 2001). However, this approach is non antigen-specific and is reversible in the absence of treatment. Novel approaches include reinstitution of control on self-antigen presentation, derived from regulatory T cells (Treg). Suppression of immune effector cells by a variety of described Treg is a key mechanism for peripheral tolerance (Maloy and Powrie, 2001). However, for control of preexisting autoimmune or other immune responses, it will be important to understand the major determinants of regulation of antigen presentation by APC.

The complex interactions resulting in the generation of T cell-mediated immune responses are dependent on antigen presentation, cognate interactions between T cells and antigen-presenting cells (APCs) including dendritic cells (DCs), and the concomitant production of soluble and membrane costimulatory molecules by APCs and T cells. DCs are implicated in the establishment and maintenance of self tolerance—both centrally and in the periphery. The molecular mechanisms controlling DC function in tolerance and immunity are poorly defined. However, B cells deprived of signaling through CD40 have been shown to induce T cell tolerance (Buhlmann et al., 1995; Hollander et al., 1996). A role for DCs in the induction of peripheral tolerance through Treg has been supported by several recent studies. The ability of myeloid DC to induce immunity or tolerance appears to be linked to its maturation state (Dhodapkar et al., 2001; Jonuleit et al., 2000; Lutz et al., 2000; Mehling et al., 2000). Immature DCs generated from murine BM induced T cell unresponsiveness in vitro and prolonged cardiac allograft survival in a preventive model (Lutz et al., 2000). Immature myeloid DCs induced $CD4^+$ Treg in vitro and $CD8^+$ Treg in vivo which each produced high levels of IL-10 and low levels of IFN-γ but no IL-4 (Dhodapkar et al., 2001; Jonuleit et al., 2000). Various drugs and cytokines, and inhibitors of NFκB have been shown to inhibit myeloid DC maturation (de Jong et al., 1999; Griffin et al., 2001; Hackstein et al., 2001; Lee et al., 1999; Mehling et al., 2000; Steinbrink et al., 1997; Yoshimura et al., 2001). DCs generated in the presence of these agents altered T cell function in vitro and in vivo, including promotion of allograft survival (Giannoukakis et al., 2000; Griffin et al., 2001). Despite this, suppression of previously primed $CD4^+$ T cell responses by DCs in vivo has not been demonstrated. This is important for therapy of preexisting autoimmune disease as $CD4^+$ effector T cells mediate the perpetuation of tissue damage in autoimmune disease through their interaction with monocytes, B cells, and local DCs (Feldmann, 2001).

NFκB activity leads to transcription of a number of genes involved in the immune response. RelB activity is required for myeloid DC differentiation (Burkly et al., 1995; Weih et al., 1995; Wu et al., 1998). In particular, we have shown that RelB regulates B cell APC function through regulation of CD40 and MHC molecule expression (O'Sullivan et al., 2000). Therefore, we hypothesized that deficiency of RelB activity in DCs would be sufficient for suppression of immune responses by myeloid DCs.

Results

Inhibition of Myeloid DC Differentiation and Antigen-Specific Suppression of Primed Immune Responses by Inhibition of RelB Function

Translocation of the NFκB family members RelB and p50 from cytoplasm to nucleus is required for myeloid DC maturation. To assess the relationship between RelB, differentiation, and tolerance induction by myeloid DCs, BMDCs were generated from homozygous $H\text{-}2^b$ $RelB^{-/-}$ or wild-type $H\text{-}2^b$ mice. $RelB^{-/-}$ BMDCs expressed no CD40 and expressed lower levels of MHC class II and CD86 than $RelB^{+/+}$ BMDCs (Figure 1A). The ability of s.c. adoptively transferred methylated BSA (mBSA)-pulsed $RelB^{-/-}$ BMDCs to prime an antigen-specific T cell proliferative response in naive wild-type $H\text{-}2^b$ mice was reduced, compared with mBSA-pulsed $RelB^{+/+}$ BMDCs (Figure 1B). To test tolerance induction, wild-type $H\text{-}2^b$ mice were injected s.c. with 5×10^5 KLH-pulsed DCs 7 days before or 7 days after priming with

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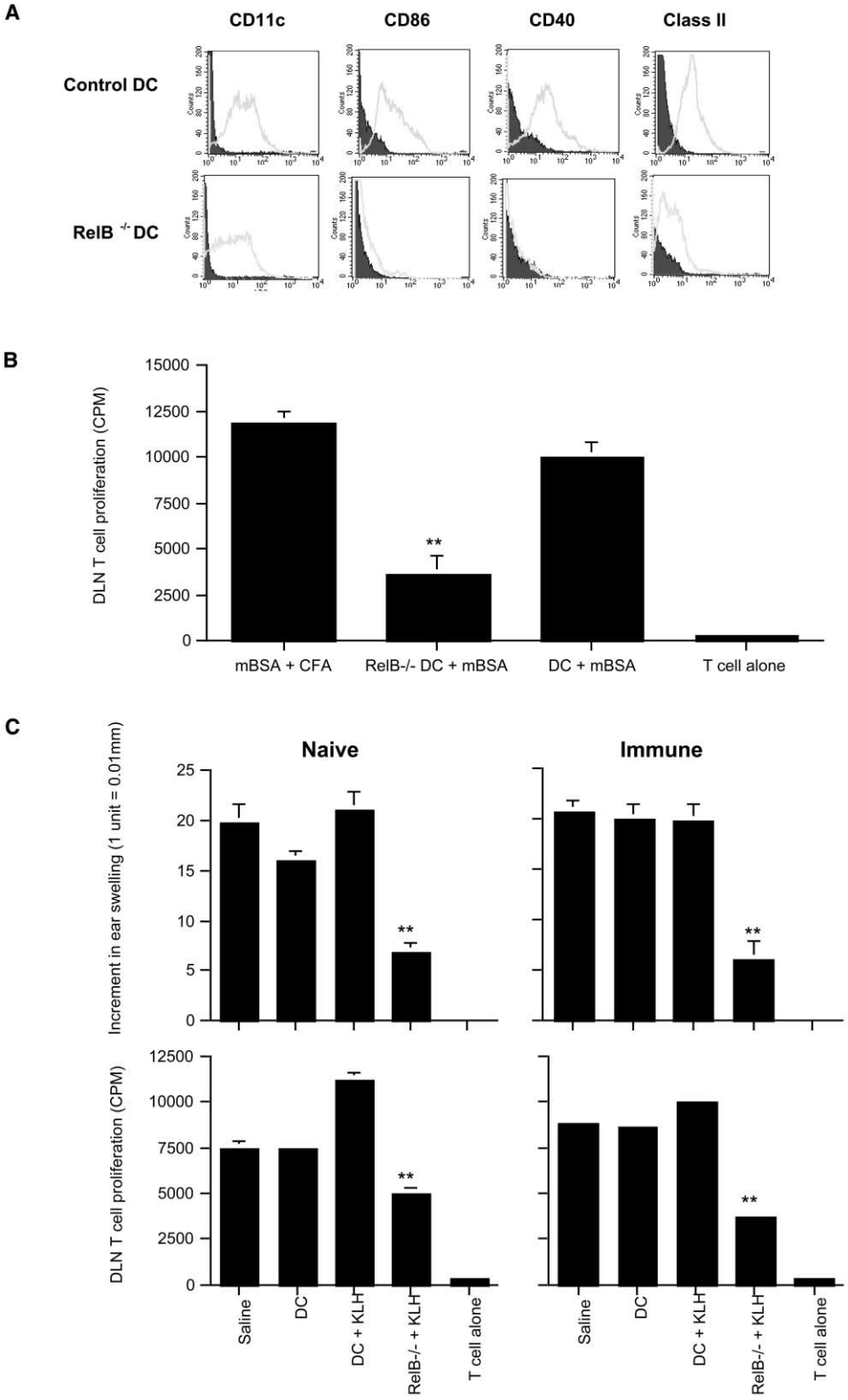

Figure 1. Suppression of Primed Immune Responses by RelB-Deficient BMDCs

BMDCs were generated from wild-type or RelB$^{-/-}$ mice, and cell surface markers were analyzed by flow cytometry (A). Wild-type mice were injected with BMDCs or mBSA in CFA as shown, and DLN mBSA-specific T cell proliferation was examined 7 days later (B). Wild-type mice were injected with BMDCs or saline as shown, 7 days before or after immunization with KLH in CFA (C). DLN KLH-specific T cell proliferation and ear KLH-specific DTH responses are shown. Mean ± SEM cpm from groups of five mice tested individually are shown. Results are representative of two separate experiments. ** $p < 0.01$, compared with wild-type DCs + mBSA.

KLH in CFA. KLH-specific immunity was tested 5 days after DC or KLH administration. Administration of DCs generated from RelB$^{-/-}$ BM not only prevented priming of specific immunity by subsequent administration of KLH/CFA, but also suppressed a previously primed immune response when administered 7 days after immunization with KLH/CFA (Figure 1C). In contrast, no suppression of KLH-specific immunity was observed following administration of KLH-pulsed RelB$^{+/+}$ DCs, whether these DCs were administered prior to or following immunization (Figure 1C).

To independently assess the relationship between RelB nuclear translocation and tolerance induction, BMDCs were generated in the presence or absence of the compound BAY 11-7082 (BAY). BAY has been shown to block TNF-α-stimulated NFκB translocation through inhibition of IκBα phosphorylation (Pierce et al., 1997). Murine BM precursors were incubated for 6 days with GM-CSF and IL-4 to produce BMDCs, in the presence or absence of BAY. All NFκB subunits were demonstrable in BMDCs generated in the absence of BAY and were present in both nuclear and cytoplasmic extracts (Figure 2A). In contrast, BMDCs generated in the presence of BAY demonstrated NFκB subunit immunoreactivity in the cytoplasm but not the nucleus (Figure 2A). BMDCs generated in the presence of BAY were similar in phenotype to RelB-deficient DCs, in that they lacked cell surface CD40 expression, and expressed reduced levels of MHC class I and class II (Figure 2B). However, CD86 was expressed at higher levels in BAY-treated DCs than RelB-deficient DCs. BMDC populations generated in the presence or absence of BAY had a dendritic morphology and expressed CD11b and low levels of F4/80 but no CD8α, CD3, CD19, Gr-1, or CD45R (data not shown). The ability of adoptively transferred BAY-treated DCs to prime an mBSA-specific T cell proliferative response in naive syngeneic mice when pulsed with the antigen mBSA was reduced, compared with mBSA-pulsed untreated BMDCs or immunization with mBSA and CFA (Figure 2C).

BAY-treated DCs were administered to mice 7 days before or 7 days after immunization with mBSA in CFA. mBSA-specific immunity was tested 5 days after DC or mBSA administration. mBSA-pulsed BAY-treated DCs prevented priming and conferred suppression of mBSA-specific immunity when compared to mBSA-pulsed DCs that had not been exposed to BAY (Figures 3A and 3B). mBSA-specific T cell proliferation, Ab production, and DTH responses were each suppressed after administration of antigen-exposed BAY-treated DCs. The data indicate that DCs in which RelB function is inhibited lack CD40, prevent subsequent priming of immunity, and suppress a previously primed immune response in vivo.

The antigen specificity of suppression and the effect of dose and route of immunization were tested by comparing the DTH responses to KLH and to mBSA after administration of varying doses of mBSA-pulsed BAY-treated DCs given i.v. or s.c. Mice injected with mBSA-pulsed BAY-treated DCs were tolerant to subsequent priming with mBSA in CFA, in a route-independent fashion, and greater numbers of DCs were more effective at inducing tolerance (Figure 3C). There was no reduction in KLH DTH responses in mice preinjected with 5 $\times$ 10^5 mBSA-pulsed BAY-treated DCs and subsequently primed with KLH in CFA (Figure 3D). The data indicate that the tolerance induced by BAY-treated DCs is DC dose dependent and specific for the antigen to which the DCs have been exposed. The lack of suppression

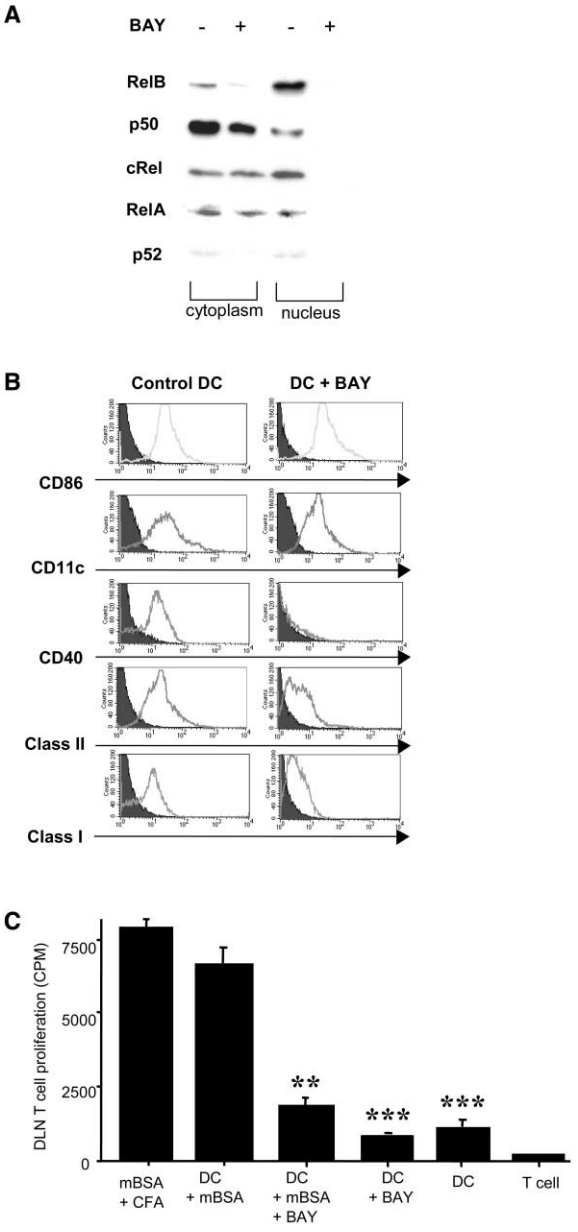

Figure 2. DC Differentiation in the Presence of an Inhibitor of NFκB Translocation Inhibits CD40 Expression and APC Function

BMDCs were generated in the presence or absence of BAY 11-7082. Nuclear and cytoplasmic extracts were immunoblotted for NFκB subunits as shown (A). Cell surface marker expression was analyzed by flow cytometry (B). Naive C57BL/6 mice were injected s.c. as shown. DLN T cell proliferation in vitro in response to exogenous mBSA is displayed as mean $\pm$ SEM cpm of triplicates of five mice assayed individually (C). Results are representative of three separate experiments. ** $p < 0.005$, *** $p < 0.001$ compared with DCs + mBSA.

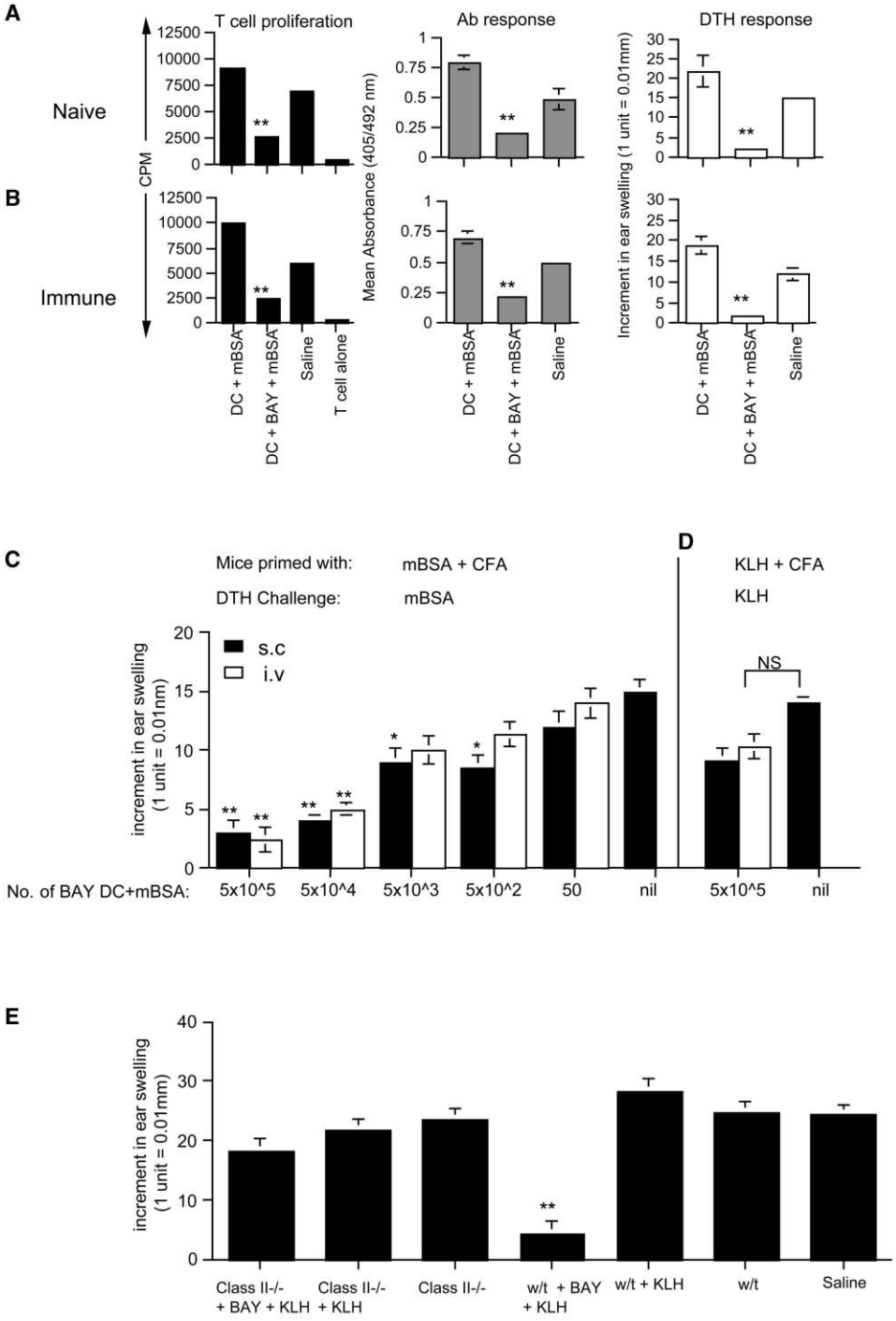

Figure 3. Suppression of Primed Immune Responses by Inhibition of RelB Function of DCs

Mice were injected s.c. with BMDCs or saline as shown, 7 days before (A) or after (B) priming with mBSA in CFA. 5 days later, mice were individually tested for DLN antigen-specific T cell proliferative, serum antibody, and ear DTH responses.

7 days after s.c. or i.v immunization with varying doses of mBSA-pulsed BAY-treated BMDCs or no DCs, mice were injected with mBSA in CFA (C) or KLH in CFA (D). DTH responses to mBSA or KLH were measured 5 days later. (E) 5×10^5 KLH-pulsed H-2^b wild-type or MHC class $II^{-/-}$ BMDCs generated in the presence or absence of BAY were administered to wild-type mice 7 days after immunization with KLH in CFA. KLH-specific immunity was tested 5 days after DC administration. Results are displayed as the mean ± SEM for each group (n = 5), tested separately, and are representative of three separate experiments. NS, not significant; * $p < 0.01$, ** $p < 0.001$.

of the KLH DTH response also excludes carry over of nonspecific suppressive effects by residual soluble inhibitor to draining LN lymphocytes.

While injected DCs are likely to present antigen directly to T cells in draining LN, it was possible that antigen-exposed injected DCs could be crosspresented

by host DCs in recipient mice. To address this, 5×10^5 KLH-pulsed H-2^b wild-type or MHC class $II^{-/-}$ BMDCs generated in the presence or absence of BAY were administered to wild-type mice 7 days after immunization with KLH in CFA. KLH-specific immunity was tested 5 days after DC administration. KLH-pulsed BAY-treated wild-type DCs conferred suppression of KLH-specific immunity when compared to KLH-pulsed BAY-treated MHC class $II^{-/-}$ DCs or KLH-pulsed wild-type DCs that had not been exposed to BAY. Neither KLH-specific T cell proliferation (data not shown) nor DTH responses were suppressed after administration of antigen-exposed BAY-treated MHC class $II^{-/-}$ DCs (Figure 3E). The data indicate that MHC class II expression by the injected DCs is necessary for subsequent suppression, thereby providing evidence that injected BAY-treated antigen-exposed DCs are not crosspresented by recipient DCs.

Suppression of Primed Immune Responses by DCs Correlates with Their RelB Nuclear Binding Activity and CD40 Expression

Previously, immature BM or monocyte-derived DCs have been shown to regulate immune responses. Since DCs in which RelB nuclear translocation is inhibited suppressed a primed immune response, the capacity of DCs prepared ex vivo to suppress immunity was correlated with RelB activity in nuclear extracts. BMDCs were generated in medium containing serum and either GM-CSF and IL-4 (control DCs), GM-CSF alone ("immature DCs") (Lutz et al., 2000), or BAY, GM-CSF, and IL-4, then pulsed with KLH and injected s.c. into mice primed 7 days previously with KLH and CFA. Immature DCs expressed lower levels of CD86, CD40, and class II than control DCs. By contrast, BAY-treated DCs expressed higher levels of CD86 and reduced CD40 compared with immature DCs (Figure 4A). Suppression by DCs of KLH-specific draining LN T cell responses correlated with the binding capacity of RelB and p50 in nuclear extracts to an NFκB consensus oligonucleotide, and with CD40 expression (Figure 4). Immunocytochemical staining of DC populations showed that the proportion of DCs that had translocated RelB to the nucleus within each population correlated with total RelB and p50 binding capacity (data not shown).

CD40 Deficiency Is Sufficient to Confer Suppression of Immunity by DCs

Since RelB-deficient and BAY-treated BMDCs lacked cell surface CD40, and suppression of immunity by DCs correlated with CD40 expression, we determined whether lack of CD40 expression by antigen-exposed BMDCs was sufficient for suppression of previously primed immunity. DCs generated from $CD40^{-/-}$ BM in the presence or absence of BAY were similar in phenotype to RelB-deficient DCs, except for higher cell surface CD86 expression (Figure 5A). DCs generated from BM of H-2^d $CD40^{-/-}$ or $CD40^{+/+}$ mice in the presence or absence of BAY were pulsed with KLH and administered s.c. to wild-type H-2^d mice 7 days after priming with KLH in CFA. Administration of DCs generated from $CD40^{-/-}$ BM conferred equivalent suppression of a previously primed immune response to DCs generated from either $CD40^{-/-}$ or $CD40^{+/+}$ BM in the presence of BAY (Figures 5B and 5C).

In the Absence of RelB or CD40, DCs Induce $CD4^+$ Regulatory T Cells that Confer Infectious Tolerance

The systemic nature of the conferred suppression suggested that in the absence of CD40 or RelB activity, DCs might induce Treg in the recipient animal. This possibility was examined in two ways. First, we tested whether induced Treg could transfer tolerance to naive or primed recipient animals (infectious tolerance) (Cobbold and Waldmann, 1998). 5×10^5 flow cytometrically sorted $CD4^+$ or $CD4^-$ T cells derived from spleens of mice injected s.c. with KLH-pulsed BAY-treated BMDCs were transferred to syngeneic recipients primed 9 days previously with either KLH or ovalbumin and CFA. Antigen-specific T cell responses were determined 7 days after T cell transfer. Adoptive transfer of $CD4^+$ T cells derived from mice previously treated with KLH-pulsed BAY-treated BMDCs strongly suppressed the subsequent KLH-specific T cell proliferative responses in recipient mice, when compared with $CD4^+$ or $CD4^-$ T cells from mice treated with KLH-pulsed untreated BMDCs (Figures 6A and 6B). Suppression by $CD4^-$ T cells derived from mice previously treated with KLH-pulsed BAY-treated BMDCs was modest or absent over a number of experiments. The OVA-specific T cell proliferative response in the recipient mice was unaffected by T cell transfers. Since T cells were identically sorted for this group, it is unlikely that the labeling mAb themselves induced suppressive capacity by the T cells.

Second, cytokine production by $CD4^+$ T cells in LN draining the site of antigen-exposed DC immunization was compared ex vivo. When compared with the $CD4^+$ T cells in LN draining the site of antigen-pulsed wild-type DCs, a greater proportion of $CD4^+$ T cells in LN draining the site of injection of either antigen-pulsed $RelB^{-/-}$ DCs, BAY-treated DCs, or $CD40^{-/-}$ DCs produced IL-10 in response to in vitro restimulation with either PMA or with KLH (Table 1). In contrast, a greater proportion of T cells primed by KLH-pulsed wild-type DCs produced IFN-γ in response to PMA or to KLH in vitro. IL-4 production was minimal in all draining LNs (data not shown). Taken together, the data indicate that DCs lacking CD40 expression or RelB function induce the differentiation of $CD4^+$ Treg that are capable of producing IL-10 in an antigen-specific manner.

To determine whether the IL-10 produced by the $CD4^+$ Treg was responsible for the observed suppression of immunity, 5×10^5 DCs generated from BM of H-2^b wild-type mice in the presence or absence of BAY were pulsed with KLH and administered s.c. to naive IL-$10^{+/+}$ or IL-$10^{-/-}$ H-2^b mice. $CD4^+$ T cells were magnetically sorted by negative selection from recipient spleens 7 days later, and 2.5×10^5 cells were transferred to wild-type mice primed 7 days previously with KLH in CFA. Adoptive transfer of $CD4^+$ T cells derived from IL-$10^{+/+}$ mice previously treated with KLH-pulsed BAY-treated BMDCs strongly suppressed the subsequent KLH-specific DTH and T cell proliferative responses (data not shown) in recipient mice, when compared with $CD4^+$ derived from IL-$10^{-/-}$ mice treated with KLH-pulsed BAY treated BMDCs (Figure 6C). Furthermore, adoptive

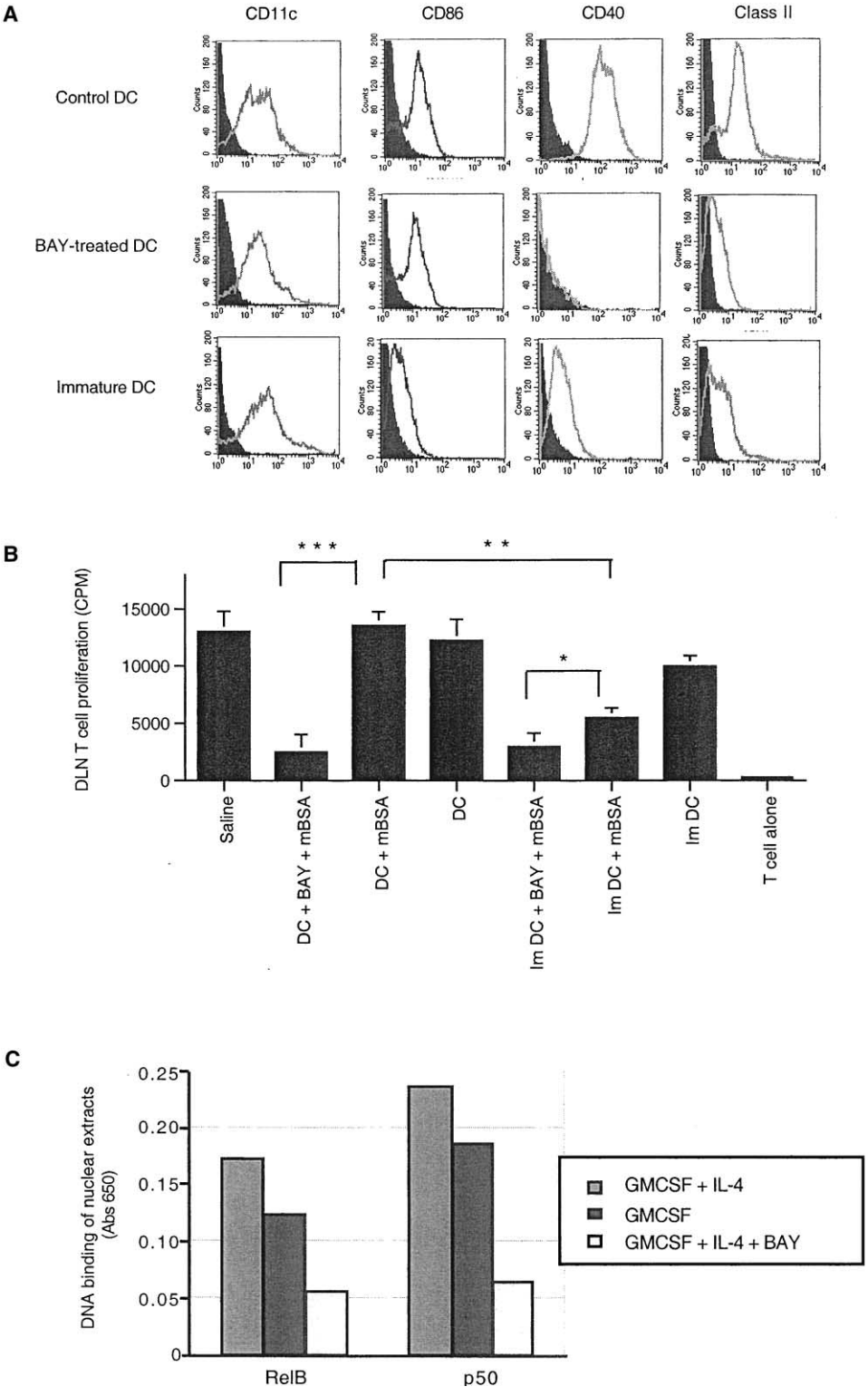

Figure 4. Suppression of Primed Responses by DCs Correlates with RelB Nuclear Binding Activity

BMDCs were generated in either GM-CSF and IL-4 (control), GM-CSF alone (immature), or GM-CSF and IL-4 in the presence of BAY, and cell surface markers were analyzed (A). Mice were primed with KLH in CFA and 7 days later injected with DCs or saline as shown. KLH-specific T cell proliferative responses were measured after 5 days (B). Nuclear extracts from DCs were bound to ELISA plates coated with NFκB consensus oligonucleotides and detected with either anti-RelB or anti-p50 (C). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.0001$.

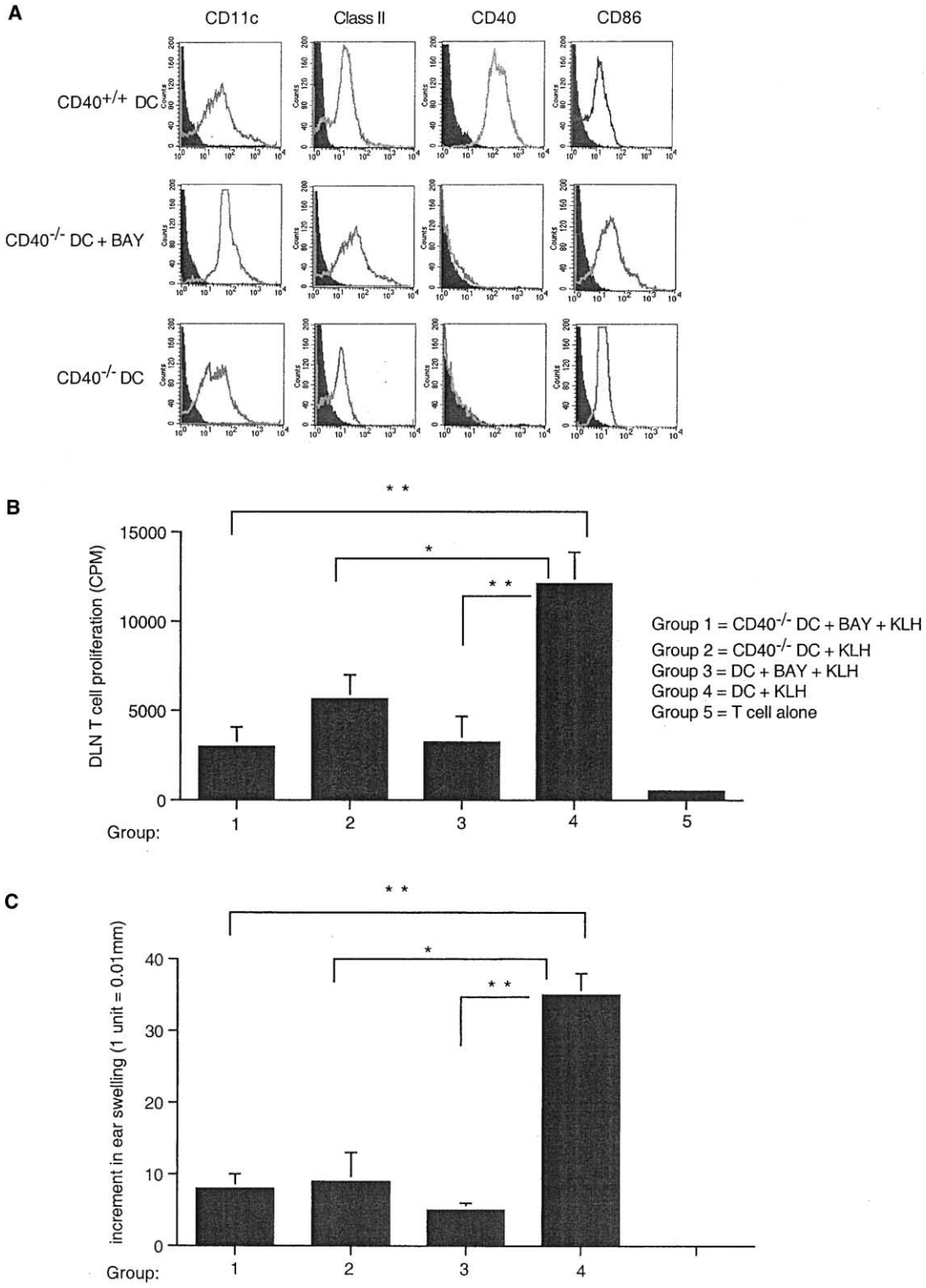

Figure 5. CD40 Deficiency Is Sufficient to Confer Suppression of Immunity by DCs

BMDCs were generated from CD40$^{-/-}$ or wild-type H-2^{d} mice in the presence or absence or BAY, and cell surface markers were analyzed by flow cytometry (A). Mice were injected with BMDCs or saline as shown, 7 days after immunization with KLH in CFA. DLN KLH-specific T cell proliferation (B) and ear KLH-specific DTH responses (C) are shown. Mean ± SEM cpm from groups of five mice tested individually are shown. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.0001$.

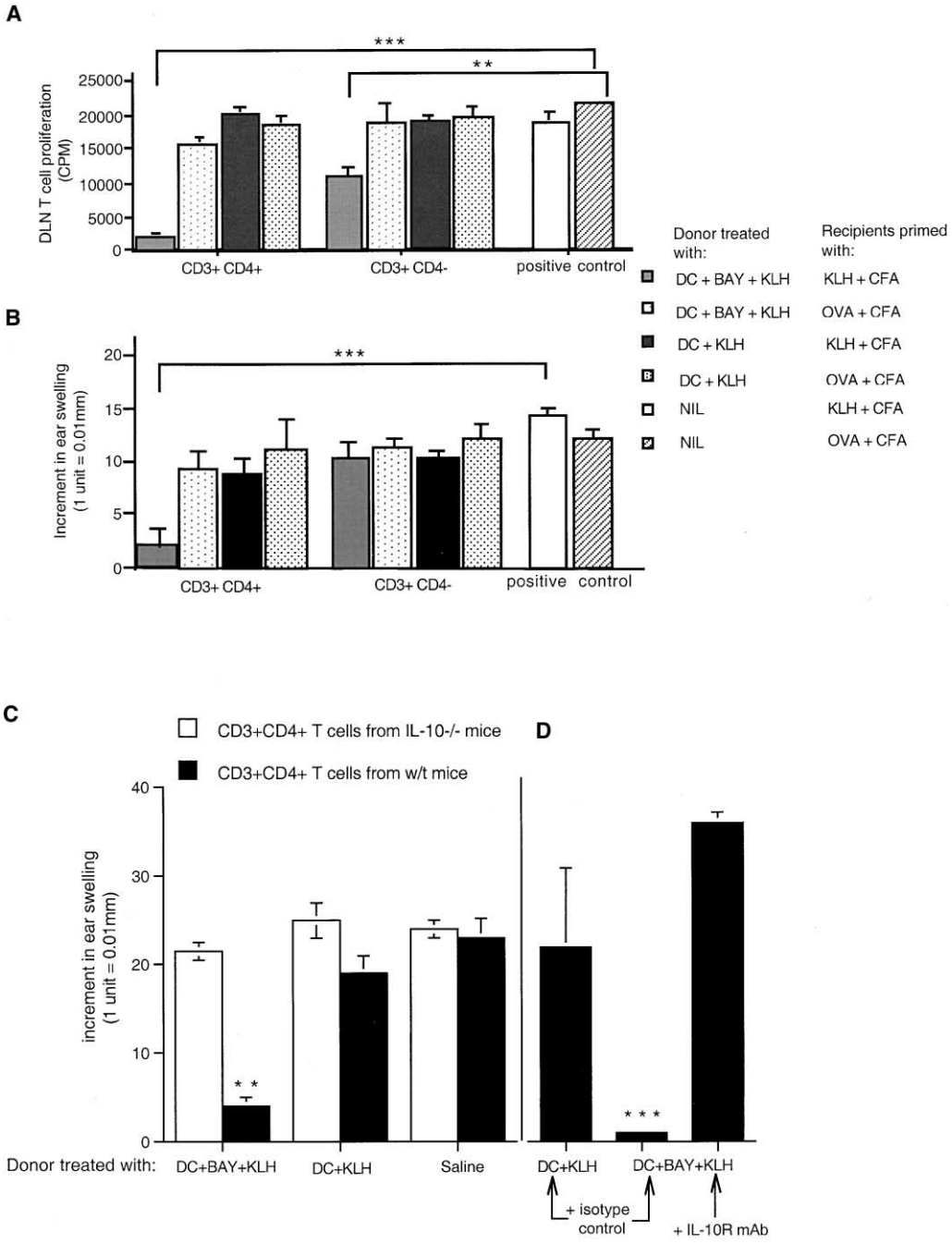

Figure 6. Antigen-Specific Tolerance Is Infectious and IL-10 Dependent

Mice were injected with 5×10^5 BMDCs as shown. Spleens were collected after 7 days, and 5×10^5 $CD4^+CD3^+$ or $CD4^-CD3^+$ cells sorted from nylon wool purified preparations were injected i.v. into nonirradiated KLH or OVA-primed syngeneic mice. Mean ± SEM cpm, KLH- or OVA-specific T cell proliferative responses in DLN (A) or DTH responses (B) measured 7 days later from groups of three mice tested individually are shown. (C and D) 5×10^5 DCs generated from BM of H-2^b wild-type mice in the presence or absence of BAY were pulsed with KLH and administered s.c. to naive IL-$10^{+/+}$ (black bars) or IL-$10^{-/-}$ (white bars) H-2^b mice. $CD4^+$ T cells were magnetically sorted by negative selection from recipient spleens 7 days later, and 2.5×10^5 cells were transferred to wild-type mice primed 7 days previously with KLH in CFA. In (D), anti-IL10R or isotype control mAb were administered i.p. to primed recipients 4 hr before T cell transfer. *** $p < 0.0001$, ** $p < 0.001$.

transfer of $CD4^+$ T cells derived from IL-$10^{+/+}$ donors previously treated with KLH-pulsed BAY-treated BMDCs strongly suppressed the subsequent KLH-specific DTH and T cell proliferative responses (not shown) in recipient mice administered isotype control mAb, but administration of IL-10R mAb to recipients blocked suppression by the T cells (Figure 6D). The data indicate that antigen-exposed DCs in which RelB function is inhibited induce

Table 1. Cytokine Production by T Cells in Draining LN

	% $CD4^+IL\text{-}10^+$ Cells	% $CD4^+IFN\gamma^+$ Cells
Ex Vivo Treatment	PMA In Vitro (mean % ± SD)	
Experiment 1, H-2^b mice		
DCs	0.62 ± 0.22	0.8 ± 0.11
DCs + KLH	0.52 ± 0.15	2.5 ± 0.62
DCs + BAY + KLH	11.23 ± 0.18	1.62 ± 0.29
$RelB^{-/-}$ DCs + KLH	3.5 ± 0.05	0.7 ± 0.11
Experiment 2, H-2^d mice		
DCs	0.9 ± 0.8	1.3 ± 0.2
DCs + KLH	1.1 ± 0.5	3.9 ± 0.8
$CD40^{-/-}$ DCs + KLH	4.7 ± 0.2	1.8 ± 0.5
$CD40^{-/-}$ DCs + BAY + KLH	5.4 ± 0.01	2.7 ± 0.2
Experiment 3, H-2^b mice	KLH In Vitro	
DCs	0.34 ± 0.32	0.98 ± 0.21
DCs + KLH	1.97 ± 0.5	3.3 ± 0.52
DCs + BAY + KLH	3.76 ± 0.3	0.4 ± 0.01
	Medium Control In Vitro	
DCs	0.1 ± 0.03	0.2 ± 0.08
DCs + KLH	0.2 ± 0.06	0.32 ± 0.07
DCs + BAY + KLH	0.12 ± 0.01	0.16 ± 0.05
	OVA In Vitro	
DCs	0.16 ± 0.01	0.19 ± 0.06
DCs + KLH	0.11 ± 0.03	0.2 ± 0.02
DCs + BAY + KLH	0.09 ± 0.02	0.1 ± 0.05

H-2^b mice were injected with KLH-pulsed DCs generated from $RelB^{+/+}$ or $RelB^{-/-}$ BM in the presence or absence of BAY (experiment 1), or H-2^d mice were injected with KLH-pulsed DCs generated from $CD40^{+/+}$ or $CD40^{-/-}$ BM in the presence or absence of BAY (experiment 2). H-2^b mice were injected with KLH-pulsed DCs generated from BM in the presence or absence of BAY (experiment 3). Control mice were injected with DCs alone. After 5 days, DLN cells were incubated in vitro with either PMA/Ionomycin (experiments 1 and 2), KLH, ovalbumin, or serum-free medium (experiment 3) for 18 hr in the presence of brefeldin A, then stained with CD4-PE and either IL-10-APC or IFN-γ-APC. Mean ± SD % cytokine-expressing $CD4^+$ T cells from groups of five mice tested individually are shown.

a population of antigen-specific $CD4^+$ regulatory T cells that regulate immune responses in an IL-10-dependent manner.

Discussion

We show here that antigen-exposed myeloid DCs, in which RelB function is inhibited, lack cell surface CD40 expression, prevent priming of immunity, and suppress a previously primed immune response. DCs in which RelB nuclear translocation is inhibited through prevention of IkB phosphorylation, DCs generated from RelB-deficient mice, and DCs generated from CD40-deficient mice similarly conferred suppression. Thus, while in vitro-derived immature BMDCs share some characteristics of the BAY-treated BMDCs, including modest induction of tolerance, CD40—as opposed to DC immaturity—determines the consequences of presentation of antigen by myeloid DCs. In this regard, DCs in which NFκB activity was suppressed during development from BM precursors, or CD40-deficient DCs expressed levels of CD86 equivalent to those of mature DCs and higher than those expressed by immature DCs. Furthermore, CD40 levels were lower than those expressed by immature BM DCs.

The current data indicate that deficiency in RelB activity leads to the generation of DCs with a unique CD40-deficient phenotype. Recently, the phenotype and viability of BMDCs generated from RelA-, c-Rel-, and p50-deficient mice were demonstrated (Ouaaz et al., 2002). BMDCs generated from RelA/p50 doubly deficient mice were more prone to death, and BMDCs generated from c-Rel/p50-deficient mice showed intact CD40 expression and APC function in MLR (when corrected for viability) but reduced IL-12 production. LPS-induced upregulation of MHC molecules, ICAM-1, CD80, and CD86 was unaffected in c-Rel/p50-deficient mice. Taken together with the current and with previous studies, these data indicate that RelA, RelB, and c-Rel—partnering with p50—each play unique and complementary roles in the process of myeloid DC differentiation (Grumont et al., 2001; Neumann et al., 2000; O'Sullivan and Thomas, 2002; Rescigno et al., 1998). In particular, RelB/p50 specifically controls functional myeloid DC differentiation and CD40 expression. A similar role for RelB and CD40 in determining the consequences of presentation of antigen by B cells has also emerged (Buhlmann et al., 1995; Hollander et al., 1996; O'Sullivan et al., 2000; Pai et al., 2002).

Induction of suppression was specific for the antigen to which DCs had been exposed. Moreover, this suppression results from induction of antigen-specific Treg, as DC administration increased the proportion of $CD4^+$ T cells producing IL-10 in draining LN, and $CD4^+$ splenic T cells from tolerant animals transferred antigen-specific tolerance to primed recipients in an IL-10-dependent manner. Therefore, the DCs induced an active infectious process of antigen-specific regulation (Cobbold and Waldmann, 1998). While the exact phenotype of the $CD4^+$ Treg induced in the current studies is not yet

elucidated, they most closely resemble Tr1 cells. Antigen-specific Tr1 cells induced in vitro produce IL-10 and suppress inflammation in colitis and allergic models in an IL-10-dependent manner (Groux et al., 1997). In keeping with the current studies demonstrating that suppression of primed immune responses by DCs correlates with their RelB nuclear binding activity and CD40 expression, human immature monocyte-derived myeloid DCs also induced $CD8^+$ T regulatory cells in vivo, which produced high levels of IL-10 and low levels of IFN-γ but no IL-4 (Dhodapkar et al., 2001).

These observations have significance for immunotherapeutic suppression of conditions in which ongoing antigen presentation is associated with chronic inflammation, including autoimmune disease, allograft rejection and graft-versus-host disease. Of importance, in the current studies induced Treg were capable of traffic from draining LN to spleen following s.c. administration of DCs. After adoptive transfer, the Treg are likely to suppress DTH responses locally in the skin, but also to suppress T cell proliferation in LN draining the site of antigen priming in the recipient animals. Further studies on the capacity of the DC-induced regulatory T cells to traffic into, and to maintain suppressive function in peripheral uninflamed or inflamed organs will be of importance in the context of autoimmune immunotherapy.

The NFκB family of proteins is regulated by IκB and other inhibitory molecules in the cytosol. Cellular activation leads to IκB phosphorylation and translocation of active NFκB to the nucleus. RelB is translocated upon myeloid DC differentiation, and it heterodimerizes with p50 in the DC nucleus (Neumann et al., 2000; O'Sullivan and Thomas, 2002; Pettit et al., 1997). RelB- and p50-deficient mice exhibit multiple deficits in immune function—in particular, RelB-deficient mice lack mature myeloid DCs and the liver and spleen are infiltrated by myeloid cells, including monocytes, granulocytes, and progenitor cells (Burkly et al., 1995; Sha et al., 1995). The role of NFκB in DC APC function was previously examined in vivo through the use of NFκB decoy oligonucleotides (Giannoukakis et al., 2000). However, in these studies NFκB inhibition was commenced several days after the initiation of the DC cultures, and CD40 expression by BMDCs was not affected by the NFκB decoy. BAY 11-7082 has been shown previously to block NFκB nuclear translocation through inhibition of IκB phosphorylation (O'Sullivan and Thomas, 2002; Pierce et al., 1997). While nuclear translocation of all NFκB subunits was inhibited in BMDCs, the specificity of the drug for NFκB is unknown. However, we provide two additional pieces of evidence that the consequences of antigen presentation by myeloid DCs are indeed determined by RelB activity. Thus, RelB-deficient BMDCs conferred similar suppression to BMDCs generated from wild-type mice in the presence of the inhibitor. Furthermore, the extent of nuclear RelB DNA binding in DCs was inversely correlated with the induction of suppression by those cells.

The lack of CD40 expressed by RelB-deficient DCs and regulation of CD40 expression by RelB transfection strongly suggested that CD40 could be a key determinant of the decision between tolerance and immunity made by a T cell in response to DCs in lymphoid organs (Diehl et al., 2000; O'Sullivan et al., 2000). On the other hand, the involvement of CD40-CD154 in priming of T cell responses to infectious organisms varies depending on the organism. Thus, Th1-mediated resistance to several organisms, including *Toxoplasma gondii*, and Th2-mediated resistance to *Schistosoma mansoni* are impaired in the absence of CD40-CD154 interactions (MacDonald et al., 2002a; Subauste et al., 1999). In contrast, CD40-CD154 appears not to be required for the Th1 response to either *Proprionibacterium acnes* or *Histoplasma capsulatum* (MacDonald et al., 2002b; Zhou and Seder, 1998). These studies strongly implicate a CD40- and NFκB-independent mechanism of DC activation utilized by certain infectious organisms. Signaling through the DAP12-associated TREM-2 receptor was recently shown to activate human myeloid DCs in an NFκB-independent manner (Bouchon et al., 2001b). Although the ligands for TREM-2 are not yet known, infectious organisms are likely candidates, based on the ability of bacteria to trigger the related TREM-1 receptor, expressed by monocytes and neutrophils (Bouchon et al., 2001a). In addition, the relevance of CD40-CD154 interactions to T cell outcome appears to be strongly influenced by the APC, in that CD40-deficient BM-derived myeloid DC and B cells are tolerogenic, whereas other splenic APC—likely a splenic DC subset—are able to stimulate allogeneic and Th2-type $CD4^+$ T cell responses in vitro (Kumanogoh et al., 2001; Ozaki et al., 1999). In this regard, DCs purified from chronically inflamed, RelB-deficient spleen, that are enriched for the $CD8\alpha$ subset, were reported to express higher levels of CD40 than DCs purified from wild-type spleen (Wu et al., 1998). Therefore, RelB-independent pathways of CD40 activation in response to inflammatory signals may particularly apply to certain splenic DC populations.

There are two broad explanations for the profound suppression of immunity associated with deficiency of CD40 expression by DC. First, generation of Treg might be a default pathway resulting from inability of CD40-deficient DCs to upregulate key costimulatory molecules, cytokines, or chemokines in response to CD40L (Cella et al., 1996). Alternatively, T cells signaled by CD40-deficient DC might provide feedback non-CD40-mediated signaling of the DC, thereby inducing other APC-derived suppressive membrane or secreted molecules. The latter model is conceptually appealing for understanding the mechanism of suppression of a previously primed immune response. Whichever explanation is correct, presentation of peptide to $CD4^+$ T cells in the absence of CD40 might represent a general mechanism of tolerance employed by several APC, including myeloid DC and B cells (Buhlmann et al., 1995; Hollander et al., 1996). Signaling of RelB through CD40 ligation and induction of CD40 expression through RelB activity represent an important feedback loop for the control of antigen presentation (O'Sullivan et al., 2000; O'Sullivan and Thomas, 2002). Similarly, inhibition either of CD40/CD154 signaling or of RelB/p50 activity in APC may have potent tolerogenic effects. For example, blockade of CD40/CD154 interactions in vivo or use of CD154-deficient recipients can prolong the life of allografts and reduce severity of autoimmune diseases (Gao et al., 1999; Hanninen et al., 2002). Furthermore, the capacity of various drugs and cytokines to inhibit NFκB provides a common mechanism linking their ability to retard the

differentiation of DCs (de Jong et al., 1999; Gregori et al., 2001; Mehling et al., 2000). In some cases, the resulting DCs also prolong allograft acceptance in vivo (Gregori et al., 2001; Griffin et al., 2001).

These data imply that the capacity of DCs prepared ex vivo to suppress or prevent immunity can be predicted based on the frequency of nuclear RelB$^+$ DCs within that population. By contrast, conditioning treatments that maximize and sustain RelB translocation, such as CD40 ligation and exposure to certain bacteria and viruses, would be predicted to stimulate Th1 and CTL responses most effectively (Hofer et al., 2001; O'Sullivan and Thomas, 2002; Ridge et al., 1998). The important role played by RelB in immune responses is exemplified by susceptibility of RelB-deficient mice to bacterial and parasitic infection, lack of Th1 responses to viral infection, and reduced susceptibility of RelB-deficient BM chimeras to inflammatory arthritis (Caamano et al., 1999; Lawlor et al., 2001; Weih et al., 1997). The relatively weak ability of immature DCs to suppress previously primed immune responses likely relates to a variable proportion of nuclear RelB$^+$ DCs within the preparations, depending on the methodology used. Furthermore, populations of immature DCs may be less likely to remain in an immature state after administration to previously primed animals, particularly at inflammatory sites, as might occur in autoimmune disease (Pettit et al., 2000). The current data highlight the potential for development of antigen-specific autoimmune immunotherapy using DCs treated with soluble inhibitors of NFκB, in view of the potent suppression of CD40 expression without the need for genetic manipulation of DC and the profound effect on previously primed immune responses in vivo.

As well as the immunotherapeutic possibilities, the current studies also suggest a mechanism by which peripheral tolerance might be constitutively maintained by myeloid DCs in which RelB and CD40 are either suppressed or not induced. We have previously demonstrated that, in contrast to inflamed or lymphoid tissues, nuclear RelB$^+$ cells are absent in noninflamed, nonlymphoid peripheral tissues (Pettit et al., 2000, 1997; Thompson et al., 2002). Therefore, at sites of antigen uptake, myeloid immature DCs lack RelB. Furthermore, a proportion of unmanipulated DCs in both periphery and LN has been shown to express little if any CD40 constitutively and to rely upon infectious signals for CD40 induction and IL-12 production (Schulz et al., 2000). Since DCs containing apoptotic bodies can constitutively migrate from peripheral sites to draining LN, myeloid DCs may reach LN lacking RelB activity and cell surface CD40 (Huang et al., 2000). Crosspresentation of processed self-antigen derived from somatic cells could thereby constitutively induce self-antigen-specific Treg in LN draining peripheral tissues. Thus, upstream signaling of DCs by infectious antigen through TLR and other pattern recognition receptors that leads to RelB translocation and CD40 induction provides a key pathway for discrimination by the immune system of antigens loaded by myeloid peripheral tissue DCs.

Experimental Procedures

Culture Medium

DCs were cultured in RPMI plus 10% heat-inactivated fetal calf serum (FCS, CSL, Parkville, Australia), supplemented with 100 μg/ml penicillin, 100 μg/ml streptomycin, 10 mM sodium pyruvate, 20 mM HEPES, 2 mM L-glutamine, and 50 μM 2-mercaptoethanol (culture medium, CM). In all experiments where mBSA is used as the challenge antigen, DCs were cultured in serum-free Excell 620 culture medium (CSL Biosciences), supplemented as above.

Bone Marrow-Derived Dendritic Cells (BMDCs)

Bone marrow cells were collected and suspended from murine long bones and passed through nylon mesh, and mononuclear cells were separated by ficoll gradient centrifugation. Macrophages, class II$^+$ cells, and lymphocytes were immunodepleted using appropriate mAb followed by magnetic beads (MACS, Miltenyi Biotec, CA). BM cells were incubated for 6–8 days in CM supplemented with 10 ng/ml each GM-CSF and IL-4 (Peprotech, USA), with fresh medium applied alternate days. DC preparations routinely contained 80%–90% CDIIc$^+$ cells. Some BMDCs were cultured continuously in the presence of 5 μM BAY 11-7082 (BioMol, Plymouth Meeting, PA) and washed three times before use. For immature BMDCs, identically treated BM cells were incubated for 8 days in CM, supplemented with 0.5 ng/ml GM-CSF.

Flow Cytometry

BMDCs were incubated for 30 min on ice with anti-CD86-FITC (GL1), anti-CD11c-PE (HL3), anti-CD11b-PE (M1/70), CD8α-PE (53-6.7), CD3-PE (17A2, all from PharMingen San Diego, CA), F4/80-PE (Serotec, Raleigh, NC), or with anti-CD40 (3/23), anti-MHC class II (2G9, both from PharMingen), anti-CD19 (1D3, American Type Culture Collection, ATCC), anti-Ly6-g and c (Gr1, RB6-BC5), or anti-CD45R (B220, RA3-6B2, each a gift from G. Hill, Queensland Institute for Medical Research, Brisbane, Qld), and followed by biotinylated rabbit anti-rat Ig (DAKO, CA) and then streptavidin-FITC (DAKO). Cells were analyzed using a FACScalibur (BD-PharMingen). Intracellular cytokine production was measured in lymphocytes by flow cytometric staining as described, with minor modifications (Sander et al., 1991). In brief, lymphocytes were stimulated in the presence of antigen or PMA/Ionomycin and brefeldin A for 18 hr, then stained with FITC-CD4, followed by 4% paraformaldehyde fixation and permeabilization with saponin (Sigma, MO). Permeabilized cells were stained with allophycocyanin (APC)-labeled anti-IL-10, APC-anti-IL-4, or APC-anti-IFN-γ (all mAb from Pharmingen) for 30 min on ice.

Protein Extraction and Immunoblotting

Nuclear and cytoplasmic extracts were prepared as previously described (Pettit et al., 1997) and protein estimated using a Protein Assay kit (Bio-Rad, Hercules, CA). 10 μg of protein extract was separated by 8% SDS-PAGE. Following transfer to nitrocellulose (Amersham, Sussex, England), membranes were immunoblotted with either anti-RelB (sc-226), anti-p50 (sc-7178), anti-c-Rel (sc-71), anti-RelA (sc-109), or anti-p52 (sc-298) antibodies (all from Santa Cruz Biotechnology), followed by sheep anti-rabbit HRP-conjugated Ig (Silenus, Hawthorn, Australia) and then detected by enhanced chemiluminescence (ECL, Life Technologies, MO).

NFκB Binding ELISA

p50 and RelB DNA binding was detected by ELISA using a Mercury Transfactor p50 Kit (Clontech, CA). 10 μg of nuclear extract was bound to wells coated with NFκB consensus oligonucleotide, incubated with either anti-RelB or anti-p50 followed by anti-rabbit HRP-conjugated Ig (Silenus), and then detected by measuring color development of TMB at 650 nm using a Multiskan plate reader (Labsystems).

Mice and Immunization

C57BL/6 and BALB/c mice (Animal Resource Centre, Perth, Australia) were maintained in specific pathogen-free (SPF) conditions. The RelB mutant C57BL/6 mice were originally generated in D. Lo's laboratory (Burkly et al., 1995). They were bred under SPF conditions in the animal facility of the Walter and Eliza Hall Institute (WEHI). Homozygous RelB$^{-/-}$ mice were selected and supplied by Dr L. Wu (WEHI) at 5–7 weeks of age for BMDC generation. CD40-deficient mice (Kawabe et al., 1994) were crossed for over ten generations under SPF conditions with BALB/c mice at the animal facility at Australian National University (ANU), and homozygous CD40$^{-/-}$

mice were supplied. IL-10 and MHC class II-deficient C57Bl/6 mice were bred under SPF conditions and supplied from ANU. All s.c. injections were delivered to the tailbase. Mice were immunized s.c. with 60 μg of mBSA or 50 μg of keyhole limpet hemocyanin (KLH) or 50 μg of ovalbumin in complete Freunds adjuvant (CFA). Serum antigen-specific Ab, draining lymph node (DLN) T cell proliferative responses, and DTH responses were measured. For the DTH responses, mice were injected i.d with either 5 μg of antigen or saline into the ears and ear swelling was measured and scored 24 hr later using an engineer's micrometer. For the adoptive transfer experiments, C57BL/6 mice were injected s.c. with 5×10^5 KLH-pulsed BMDCs. Spleens were removed 7 days later, and splenocytes were enriched for T cells by transfer to sterile nylon wool columns (Robbins Scientific, Sunnyvale, CA) for 1 hr at 37°C. In some experiments, $CD3^+CD4^+$ and $CD3^+CD4^-$ cells were sorted, after staining with anti-CD3-FITC and anti-CD4-PE, using a Moflo flow cytometer (Cytomation, Fort Collins, CO). Purity was approximately 85%. In other experiments, $CD3^+CD4^+$ T cells were purified by immunomagnetic depletion with anti-CD8, anti-MHC class II, Gr1, B220, and F4/80. $2.5–5 \times 10^5$ of each purified population was injected i.v. into nonirradiated syngeneic recipients, primed 7–9 days previously with either KLH or ovalbumin in CFA. Antigen-specific T cell proliferative responses were measured in DLN after 7 days. For in vivo mAb blocking studies, recipient mice were administered 1 mg anti-IL-10R mAb (1B1.3a, PharMingen) or isotype control mAb i.p. 4 hr before transfer of $CD3^+CD4^+$ T cells.

In Vitro Proliferative and Antibody Responses

For the T cell proliferation assay, a single-cell suspension was prepared from the inguinal LNs. 4×10^5 LN cells/well were incubated in triplicate in the presence or absence of varying concentrations of antigen or 1 μg Concanavalin A (Sigma), at 37°C in 5% CO_2 for 3 days. In all experiments where mBSA is used as the challenge antigen, assays were carried out in serum-free Excell 620 culture medium. Cells were pulsed with 1 μCi ^{3}H-thymidine/well for the final 6–8 hr, then harvested onto glass fiber filters using an automated cell harvester. Incorporated ^{3}H-thymidine was counted using a Packard TopCount NXT (Packard, Meriden, CT). Specific [^{3}H]-thymidine incorporation (cpm) was the mean ± SEM of triplicate wells.

For mBSA or KLH-specific Ab determination by ELISA, serum was prepared from lateral tail vein blood. 100 μl of mBSA or KLH protein, at 10 μg/ml in 50 mM carbonate buffer (pH 9.6), was coated onto the wells of 96-well microtiter plates (Griener Labortechnik, Kresmuster, Austria). After washing with 0.5% Tween 20/PBS and blocking with 200 μl 3% BSA fraction V, 100 μl serum in 5-fold dilutions was added to duplicate wells. After washing, each well was incubated with 100 μl of biotinylated rabbit anti-mouse Ig secondary antibody (DAKO), followed by washing and incubation with streptavidin-horseradish peroxidase. After incubation with 0.1% ABTS in 0.03% hydrogen peroxide and 150 mM citrate buffer (pH 4.5), the presence of antigen-specific antibodies was detected by the net absorbance readings at 405 nm and 492 nm.

Statistical Analysis

Differences were compared using Student's t tests. Differences were considered significant at $p < 0.05$.

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